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ALGINATE MATRICES PREPARED IN SUB AND SUPERCRITICAL CO_2

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This study deals with the preparation and characterization of calcium alginate porous matrices obtained by ionically crosslinking with calcium ions of the frozen sodium alginate solution pretreated in sub and supercritical CO_2 in absence of co-solvents. Porous matrices from natural biopolymer alginate extracted from brown seaweeds have biomedical applications such as drug delivery and cell culture. The encapsulation efficiency depends on the foaming and the crosslinking method of biopolymeric matrices. High porous and reliable matrices are obtained by foaming with CO_2 at high pressure in sub and supercritical treatment of sodium alginate solutions. In this contribution are shown the conversion of sodium and calcium alginate solution in high porous –crosslinked matrices were by foaming in CO_2 performed at 25–40^{0}C and 1, 80, and 100 bar. The structural characterization of the samples are analyzed by circular dichroism to estimate the ratio mannuronate to guluronate residues. The effect of pressure, temperature, processing time (20 minutes and 5 hours), and the presence of glycerol on the structure of calcium alginate matrices were investigated by optical microscopy and differential scanning calorimetry.

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1. Introduction

Porous matrices of natural and synthetic polymers have applications such as scaffolds for cell and tissue transplantation and they are investigated as matrices for the entrapment and/or releasing of biological agents. Among polymers, alginate has several properties appropriate for medical applications as cell encapsulation and drug controlled releasing. Alginate is a natural polysaccharide extracted from brown marine algae seaweed and from bacteria. It is a linear unbranched copolymer containing β (1-4)-linked D-mannuronic acid and α (1-4)-linked L-guluronic acid residues. Alginates consist of blocks of similar (MM or GG) or alternating (MG) residues, number and distribution of monomers depending on the species and the age of the alginate source. They are readily produced in aqueous solution of sodium alginate cross-linked in the presence of multivalent cations such as Ca^{2+}, Ba^{2+} or Sr^{2+} becoming a flexible gel matrix. According to circular dichroism measurements, it has been established that calcium ions react preferentially with the L-guluronic segments of alginate forming an egg-box structure [1]. In such way the hydrophilic alginate gel with low toxicity is used as a matrix support material for tissue engineering with the aim of therapeutic reconstruction of damaged tissue, to entrap molecules such as proteins and enzymes and also for the controlled release of drugs [2-6]. Scaffolds made of such alginate gels used as ma-

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trices have limited properties such as biocompatibility, biodegradability with proper degradation rate due to high non-uniform size and uncontrollable porosity.

In order to produce alginate matrices with high homogeneity in texture, the polymer foaming has been performed in supercritical carbon dioxide. Supercritical carbon dioxide can be regarded as a good alternative solvent for polymer processing due to its desirable physical and chemical properties such as relatively chemical inertness, readily accessible critical point ($T_c = 31.1^0C$, $P_c = 73.8$ bar), very good wetting characteristics, low viscosity, highly tunable solvent behavior, due to combination of gas-like diffusivity and liquid-like density which can be tuned by modifying the pressure. Moreover, supercritical CO_2 is a non-toxic, non-flammable, inexpensive and readily available gas with high diffusion rate, low viscosity and no surface tension [7].

The plasticizer, glycerol, improves mechanical properties and texture for scaffolds. The addition of a plasticizer is essential. Adding a plasticizer consisting of small molecules with low volatility, to a polymeric matrix will produce a modification of the three-dimensional structure and changes the functional properties of matrix by increasing dispensability, extensibility and flexibility and decreasing cohesion, mechanical properties, elasticity and rigidity [8, 9]. Partap et al. prepared porous calcium alginate in water/bis(2-ethylhexyl sulfosuccinate) sodium salt/isooctane/super-critical CO_2 emulsions where CO_2 plays a dual role: as a reagent increasing the acidity of the aqueous phase containing insoluble $CaCO_3$ and as a foaming agent [10].

The aim of this study was the obtaining of calcium alginate matrices with uniform texture using high pressure CO_2 as foaming agent without co-solvents. Sodium alginate solutions were processed in high pressure CO_2, with freezing. After depressurization the frozen samples were ionically crosslinking with calcium ions. The effects of the presence of glycerol as plasticizer, carbon dioxide pressure, temperature, and processing time (20 minutes and 5 hours) on the structure of the obtained calcium alginate matrices were investigated.

2. Experimental

2.1. Materials

Alginic acid sodium salt from brown algae, supplied from Fluka, calcium chloride and glycerol from Merck. Pure carbon dioxide (99.998%), purchased from Linde Gas Romania.

2.2. Synthesis of Ca-alginate matrices

Porous marine polysaccharide calcium alginate matrices were prepared from sodium alginate solution (3 wt%) with and without plasticizer (glycerol) and foaming in supercritical CO_2. After foaming in high pressure the samples were frozen at -10^0C followed by a slow release of CO_2. The frozen samples of sodium alginate were ionically cross-linked with calcium ions (5 wt% $CaCl_2$ solution). Experiments were performed at 25 and 40^0C and CO_2 pressures between 1 to 100 bar with processing time 20 minutes and 5 hours. The samples were denoted as P (bar)/T (^0C)/ processing time in CO_2 (20 min or 5 hours). If the sample contains glycerol (50% by weight of alginate), the sign Gly is added.

2.3. Characterization

The circular dichroism (CD) spectra: recorded with Jasco J-815 CD spectrometer. The absorbance of sodium alginate solutions (0.25 wt%) was measured from 190 to 250 nm at 25^0C using a quartz cell with an optical pathway of 10 mm and water as reference.

Optical micrography: microscop Bresser Biolux AL with videocamera

Differential scanning calorimetry: DSC Mettler Toledo in air at a heating rate of 10^0C/min in the range 25-250^0C. (Temperature precision ±0.02K, calorimetric data resolution 0.01 μW).

3. Results and discussions

3.1. CD spectroscopy

CD spectroscopy is used for the study of structural changes of sodium alginate after high pressure CO_2 treatment, before ionically crosslinking with calcium ions. It is a powerful method for analyzing the structure of optically active materials such as alginate, which have carboxylate as the intrinsic chromophore [11]. The CD spectrum for guluronate residues in alginate is negative while the spectrum for mannuronate residues is positive.

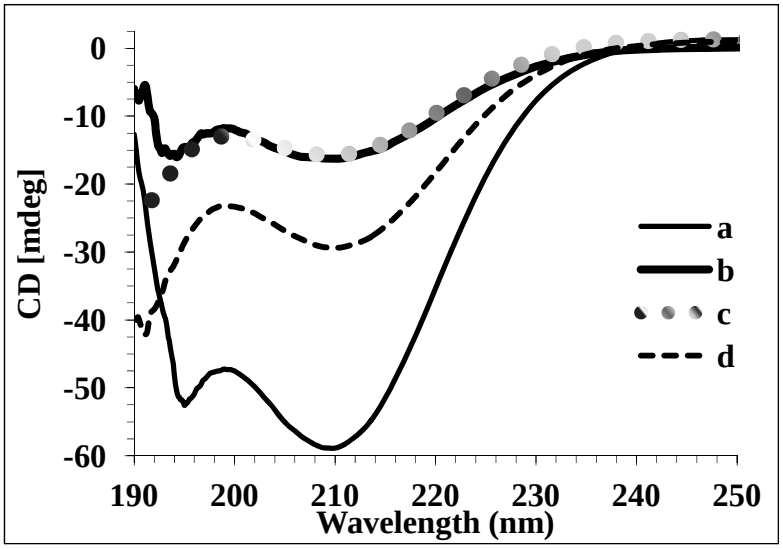


Fig. 1. CD spectra of sodium alginate solution at atmospheric pressure and 25^{0}C (a) and treated 20 min in high pressure CO_2 at 82 bar and 25^{0}C (b), 82 bar and 40^{0}C (c) and 100 bar and 40^{0}C (d).

The dichroic spectra of sodium alginate (Figure 1) show a peak at 198 nm and a trough at 210 nm which are assigned to $n \rightarrow \pi^*$ transitions of carboxylate groups. The ratio of peak height to trough depth varies with the ratio of mannuronate to guluronate residues and can be calculated using equations mannuronate/guluronate ≈ 2.0 (peak/trough) if peak/trough <1 and % mannuronate ≈ 27 (peak/trough) + 40 if peak/trough >1 [1]. The peak/trough ratios were less than one for all samples, which indicate that sodium alginate is guluronate rich. The M/G ratios are calculated and the results presented in Table 1 show that all samples have approximately the same M/G values. The treatment of sodium alginate with CO_2 at pressure and temperature up to 100 bar and 40^{0}C respectively, does not influence the mannuronate content.

Table 1. Mannuronate and guluronate content in sodium alginate samples unprocessed and treated in high pressure CO_2, 20 minutes

Sample		M/G*	G%	M%
P (bar)	T (^{0}C)			
1 (air)	25	1.54	39.4	60.6
82	25	1.52	39.7	60.3
82	40	1.56	39.1	60.9
100	40	1.50	40.0	60.0

* mean values for 3 samples

The intensity changes in dichroic spectra (Figure 1) due to CO_2 increases the acidity of the samples. The intensity of carboxylate peak for samples processed in sub and supercritical CO_2 is

lower than for sodium alginate solution obtained at atmospheric pressure and 25^0C. An isodichroic point at about 238 nm indicates equilibrium between dissociated and non-dissociated carboxyl groups. The non-dissociated carboxyl groups interact by intra and intermolecular hydrogen bonds leading to the intensity decreasing in dichroic spectrum for sodium alginate processed for 20 minutes with carbon dioxide at 82 bar and 25^0C and 40^0C respectively. At higher pressure (100 bar), CO_2 prevents the formation of these intermolecular bonds and optical activity of carboxyl groups are distinguished by a higher intensity of peak and trough in dichroic spectrum.

3.2. Optical microscopy

The optical images (Figure 2) illustrate that after the treatment of sodium alginate with CO_2 at high pressure, in the presence of glycerol, calcium alginate matrices with porous structure are obtained after crosslinking. The micrographs show that the plasticizing effect of glycerol leads to larger pores formation. The processing time also influences the matrix porosity. After 5 hours of processing in supercritical CO_2 followed by crosslinking of sodium alginate, a large-porous matrix with is obtained.

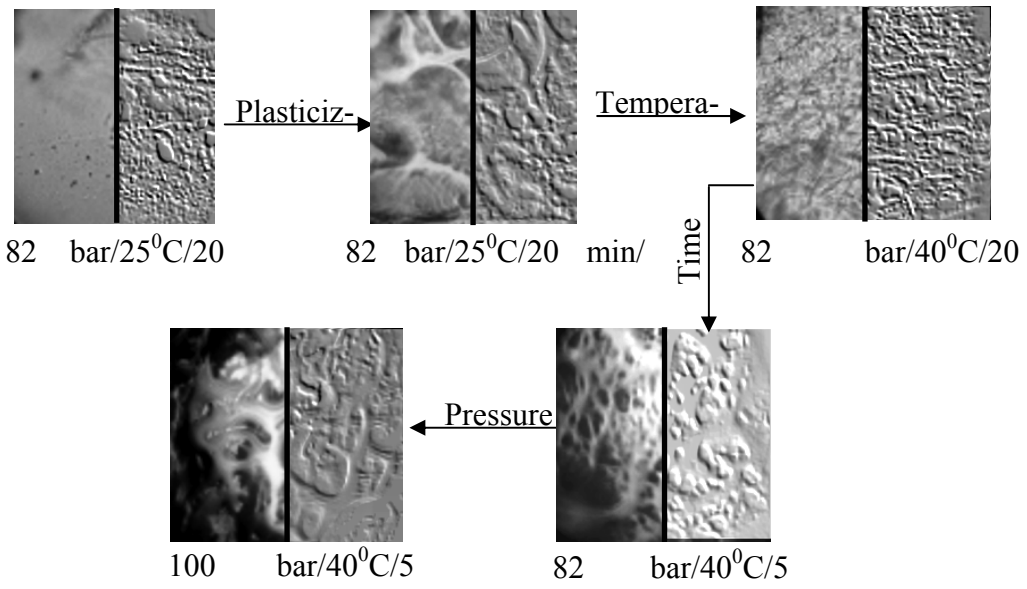
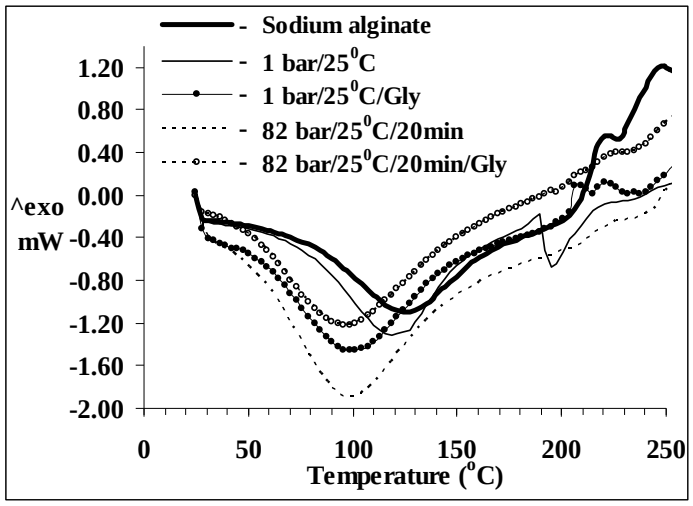


Fig. 2. Optical topography for alginate matrices: effect of glycerol, temperature, processing time and CO_2 pressure. (left-optical images (x40); right- specific features, see text)

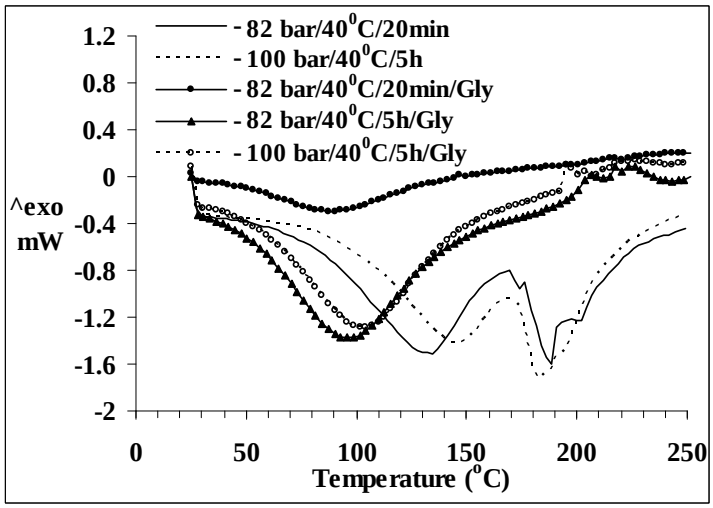
The processing time in CO_2 induces the pore growth. This is congruent with typical structure of polymers due to their foaming with gases or supercritical fluids [12-14]. The pores dimension increases with supercritical CO_2 pressure. From 82 to 100 bar, more CO_2 is dissolved into the sodium alginate aqueous solution with the formation of larger pores in the calcium alginate matrix. The alginate matrix with dense structure was obtained by crosslinking of sodium alginate solution with glycerol, which was previously processed in supercritical CO_2 (82 bar and 40^0C) for 20 minutes. The images show a network of interconnected pores for the sample 82 bar/40^0C/20 min/Gly. When the temperature increases from 25 to 40^0C, the dissociation degree of sodium alginate is high and the biopolymer becomes more hydrophilic. In this case the ionically crosslinking of sodium alginate is favorable and the obtained alginate matrix is high crosslinked.

3.3. DSC

The plasticizer effect of glycerol and thermal transitions of the biopolymeric matrices were evidenced by DSC (Figure 3).



(a)



(b)

Fig. 3. DSC curves of (a) sodium alginate powder and calcium alginate matrices unprocessed (1 bar/20^{0}C) and processed in subcritical (82 bar/25^{0}C) and (b) supercritical (82-100 bar/40^{0}C) CO_2.

The DSC curves show both endothermic peaks due to the water releasing from the calcium alginate matrices and due to the melting of the samples, and the exothermic peaks due to polymer degradation. The polymer containing hydrophilic groups usually presents strong interaction with water. In the case of alginate there are three different kinds of water which can be released: free water lost between 40 - 60^{0}C, bound water to the hydroxyl groups lost until 120^0 C and linked water to the –COO- groups lost until 160^{0}C [15]. The endothermic peaks assigned to dehydration are 124^{0}C for sodium alginate and 118^{0}C for calcium alginate. These values indicate that in sodium alginate the –carboxylate groups form hydrogen bonds with water while in calcium alginate most carboxylate groups are involved in the formation of egg-box crystalline structure, water being bonded in large proportion on hydroxyl groups. It can be concluded that the used alginate has many guluronic groups which interact with Ca^{2+}, result that confirms the conclusions obtained from CD spectroscopy data. The endothermic effect at 196^{0}C corresponds to the melting of crystalline structure of calcium alginate. The presence of glycerol leads to decreasing of dehydration temperature for all samples, well as to some exothermic effects in the temperature range 200 – 220^{0}C due to breaking the hydrogen bonds between OH groups of glycerol and carboxylate groups of alginate. The dehydration temperature decreases from 99 to 96^{0}C for samples 82 bar/25^{0}C/20 min respectively 82 bar/25^{0}C/20 min/Gly for which the plasticizer effect of glycerol was also evidenced by optical microscopy (Figure 2). Subcritical CO_2 has also a plasticizer behavior as can be

seen in DSC curve of sample 82 bar/25^{0}C/20 min by comparison with sample 1 bar/25^{0}C. The temperature increasing means a transition from subcritical to supercritical state of CO_2, therefore a different behavior in the alginate processing. The endothermic effect corresponding to the melting of crystalline structure also appears at 188^{0}C (sample 82 bar/40^{0}C/20 min) and 184^{0}C (sample 100 bar/40^{0}C/5 h) at calcium alginate matrices without glycerol, obtained from sodium alginate processed in supercritical CO_2. For these samples, dehydration takes place at 135^{0}C, respectively 146^{0}C meaning that the water is bound by ion-dipole interaction with carboxyl groups in alginate, CO_2 decreases the pH of the samples and a fraction of carboxyl groups exist in non-dissociated form. More CO_2 at 100 bar means more carboxyl groups in alginate samples leading to increase the dehydration temperature. The existence of –COOH groups in the samples processed in supercritical CO_2 is revealed also by CD spectroscopy. The exothermic degradation of sodium alginate takes place at 220°C, while calcium alginate samples do not show degradation until 250^{0}C.

4. Conclusions

Calcium alginate matrices with porous uniform texture were obtained by crosslinking frozen sodium alginate aqueous solutions, with and without glycerol, processed in high pressure CO_2 as foaming agent, with calcium ions. The porous structure of the obtained biopolymeric matrices was evidenced by optical microscope observation. The porosity increases with CO_2 pressure, processing time, and glycerol adding. Ionically crosslinking of guluronic groups of sodium alginate with calcium ions was evidenced by the appearance in the DSC curve of an endothermic effect corresponding to the melting of the crystalline structure of calcium alginate. The CD measurements show that the CO_2 processing of sodium alginate solution does not influence the M/G ratio values but increases the acidity of the samples, which lead to the appearance of non-dissociated carboxyl groups. These groups cannot form complex with Ca^{2+} but can interact through intra and intermolecular hydrogen bonds with the formation of calcium alginate matrix with compact structure observed also by optical microscopy. The presence of carboxyl groups is also evidenced in the DSC curves by increasing the dehydration temperature of the samples obtained from sodium alginate without glycerol, processed in supercritical CO_2. The presence of glycerol has a decreasing effect on dehydration temperature.

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